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U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER
4703/0J586

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

09/889313

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/00068	11 January 2000	11 January 1999

TITLE OF INVENTION

PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

APPLICANT(S) FOR DO/EO/US

Shuji KITAMURA and Takashi UEYAMA

Applicant herewith submits to the United States Designated/Elected office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. [] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S. C. 371.

3. [] This is an express request to begin national examination procedures (35 U.S.C. 371 (f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S. C. 371 (b) and PCT Articles 22 and 39 (1).

4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. [X] A copy of the International Application as filed (35 U.S. C. 371 (c) (2))
   a. [] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau
   c. [] is not required, as the application was filed in the United States Receiving Office (RO/US)

6. [X] A translation of the International Application into English (35 U.S. C. 371 (c)2)).

7. [] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. [] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [] have been transmitted by the International Bureau.
   c. [] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [] have not been made and will not be made.

8. [] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c) (3)).

9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. [] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 (with 3 references).

12. [x] An assignment document for recording. A **separate** cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. [x] A FIRST preliminary amendment.
    [] A SECOND or SUBSEQUENT preliminary amendment.

14. [] A substitute specification.

15. [] A change of power of attorney an/or address letter.

16. [x] Other items or information: Affirmation of Priority Claim

EXPRESS MAIL CERTIFICATE

Date 7/11/01 Label No. EL067446638US

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

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Name (Print)

Signature

JC18 Rec'd PCT/PTO 1 1 JUL 2001

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) 09/889313	INTERNATIONAL APPLICATION NO.: PCT/JP00/00068	Attorney's Docket Number 4703/0J586

17. [x] The following fees are submitted:		ALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492 (a)(1)-(5)): Search Report has been prepared by the EPO [X] or JPO []	**$860.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482)	**$690.00**		
No international preliminary examination fee paid to USPTO(37 CFR 4.482) but international search fee paid to USPTO (37 CFR 1.445 (a) (2)...	**$710.00**		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....	**$1,000.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....	**$100.00**		
			$860.00
Surcharge of $130.00 for furnishing the oath or declaration later than []20 []30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**ENTER APPROPRIATE BASIC FEE AMOUNT =**		$

Claims	Number Filed	Number Extra	Rate		
Total Claims	**17-20**	0	X $18.00	**$0.00**	
Independent Claims	2-3	0	X $80.00	**$0.00**	
Multiple dependent claims(s) (if applicable)		+270		$	
			TOTAL OF ABOVE CALCULATIONS =	**$860.00**	
Reduction by 1/2 for filing by small entity, if applicable.				$	
			SUBTOTAL =	**$860.00**	
Processing fee of **$130.00** for furnishing the English translation later the [] 20 [] 39 months from the earliest claimed priority date (37 CFR 1.492(f)).		+		$	
			TOTAL NATIONAL FEE =	**$860.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). the assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property		+		**$40.00**	
			TOTAL FEES ENCLOSED =	**$900.00**	
				Amount to be: refunded	$
				charged	$

a. [X] A check in the amount of $900.00 to cover the above fees is enclosed.

b. [] Please charge my Deposit Account No.04-0100 in the amount of $ to cover the above fees.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 04-0100. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:
S. Peter Ludwig, Esq.
Darby & Darby P.C.
805 Third Avenue
New York, New York 10022-7513

SIGNATURE _____

NAME **S. Peter Ludwig**

REGISTRATION NO. **25,351**

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Signature

File No.: 4703/0J586US0

DARBY & DARBY P.C.
805 Third Avenue
New York, NY 10022
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Date: July 11, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: KITAMURA et al.

Serial No: To be assigned
(U.S. National Phase of
International Application No. PCT/JP00/00068
Filed: 11 January 2000)

Filed: Concurrently herewith

For: METHOD FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND METHOD FOR PRODUCING WHEY (AS AMENDED)

PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Box PCT
Washington, DC 20231
Attn: DO/EO/US

Sir:

Prior to examination, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Please amend the title to read:

"METHOD FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND METHOD FOR PRODUCING WHEY"

IN THE CLAIMS:

Please cancel claim 2.

Amend claims 3-7 and 9 as follows:

3. (Amended) The method of claim 1 wherein said milk is selected from the group consisting of cow's milk, goat's milk, sheep's milk, soy bean milk, skim milk, reconstituted milk, powdered mil, condensed milk and mixtures thereof.

4.(Amended) The method of claim 1 wherein said fermented milk has a viscosity of not higher than 20 cp.

5.(Amended) The method of claim 1 wherein said angiotensin converting enzyme inhibitory peptide is selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro, and mixtures thereof.

6.(Amended) The method of claim 1 wherein said mixed material further contains a yeast.

7.(Amended) The method of claim 1 wherein said lactic acid bacteria contained in the mixed material comprises *Lactobacillus helveticus*.

9.(Amended) A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:

subjecting the fermented milk produced by the method of claim 1 to at least one of centrifugation and filter pressing to separate and recover whey.

Add the following new claims:

10. (New) A method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated, and

(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces and said whey containing the angiotensin converting enzyme inhibitory peptide is produced.

11. (New) The method of claim 10 wherein said milk is selected from the group consisting of cow's milk, goat's milk, sheep's milk, soy bean milk, skim milk, reconstituted milk, powdered milk, condensed milk and mixtures thereof.

12. (New) The method of claim 10 wherein said fermented milk has a viscosity of not higher than 20 cp.

13. (New) The method of claim 10 wherein said angiotensin converting enzyme inhibitory peptide is selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro, and mixtures thereof.

14. (New) The method of claim 10 wherein said mixed material further contains a yeast.

15. (New) The method of claim 10 wherein said lactic acid bacteria contained in the mixed material comprises *Lactobacillus helveticus*.

16. (New) The method of claim 15 wherein said *Lactobacillus helveticus* comprises *Lactobacillus helveticus* CM4.

17. (New) A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:

subjecting the fermented milk produced by the method of claim 1 to at least one of centrifugation and filter pressing to separate and recovery whey.

18. (New) A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:

subjecting the fermented milk produced by the method of claim 10 to at least one of centrifugation and filter pressing to separate and recover whey.

REMARKS

The title is amended to more particularly describe the subject matter that Applicant regards as their invention.

Claim 2 is cancelled. Claims 3-7 and 9 are amended to remove multiple dependency. These amendments broaden the scope of the claims and are made to avoid payment of fees for multiple dependent claims. Claim 9 is further amended to correct an obvious error. Claims 10-18 are new. No new matter is added as a result of the amendment.

A prompt official action on the merits of the claims is respectfully requested.

Respectfully submitted,

By: _____
S. Peter Ludwig
Reg. No. 25,351

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Signature

File No.: 4703/0J586US0

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805 Third Avenue
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Date: July 11, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of: KITAMURA et al.

Serial No: To be assigned
(U.S. National Phase of
International Application No. PCT/JP00/00068
Filed: 11 January 2000)

Filed: Concurrently herewith

For: METHOD FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND METHOD FOR PRODUCING WHEY (AS AMENDED)

MARK UP TO ACCOMPANY PRELIMINARY AMENDMENT

Hon. Commissioner of
Patents and Trademarks
Box PCT
Washington, DC 20231
Attn: DO/EO/US

Sir:

IN THE SPECIFICATION:

Amend the title as follows:

[PROCESS] METHOD FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND [PROCESS] METHOD FOR PRODUCING [MILK SERUM] WHEY

IN THE CLAIMS:

Amend claims 3-7 and 9 as follows:

3. (Amended) The method of claim 1 [or 2] wherein said milk is selected from the group consisting of cow's milk, goat's milk, sheep's milk, soy bean milk, skim milk, reconstituted milk, powdered mil, condensed milk and mixtures thereof.

4. (Amended) The method of claim 1 [or 2] wherein said fermented milk has a viscosity of not higher than 20 cp.

5. (Amended) The method of claim 1 [or 2] wherein said angiotensin converting enzyme inhibitory peptide is selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro, and mixtures thereof.

6. (Amended) The method of claim 1 [or 2] wherein said mixed material further contains a yeast.

7. (Amended) The method of claim 1 [or 2] wherein said lactic acid bacteria contained in the mixed material comprises *Lactobacillus helveticus*.

9. (Amended) A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:

subjecting the fermented milk produced by the method of claim 1 [or 2] to at least one of centrifugation and filter pressing to separate and recover whey[, is provided].

SPECIFICATION

PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

Field of Art

The present invention relates to a method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide which enables effective production of fermented milk containing an angiotensin converting enzyme inhibitory peptide such as Val-Pro-Pro and/or Ile-Pro-Pro, and to a method for producing whey containing an angiotensin converting enzyme inhibitory peptide which enables effective separation and production of whey containing an angiotensin converting enzyme inhibitory peptide.

Background Art

Angiotensin Converting Enzyme (abbreviated as "ACE" hereinbelow) is found mainly in lungs or vascular endothelial cells. ACE acts on angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Pro-Phe-His-Leu), which has been generated by digestion with renin, to release a dipeptide (His-Leu) from its C-terminal, thereby giving angiotensin II, which causes contraction of vascular smooth muscle and has strong hypertensive

effect. This enzyme also decomposes and inactivates bradykinin, which has hypotensive effect. Such ACE produces hypertensive peptide (angiotensin II) and at the same time inactivates hypotensive peptide (bradykinin), so that it exhibits hypertensive effect. Therefore, angiotensin converting enzyme inhibitor (abbreviated as ACEI hereinbelow), which inhibits activity of ACE, has hypertension inhibitory effect.

As ACEI, there are known peptides having three to ten amino acid residues including Val-Pro-Pro (Japanese Patent No. 2782142) and a tripeptide Ile-Pro-Pro (JP-A-3-120225). There is also known a peptide having ACEI activity, which is produced by digestion of milk casein by protease produced by lactic acid bacteria, and found in dissolved state in whey of fermented milk (J. Dairy Sci. 78, 6, p1253-1257, 1995).

Such peptides as ACEI may be taken in the form of fermented milk per se containing Val-Pro-Pro and/or Ile-Pro-Pro. However, in view of the concentration and effective dose of the peptides as ACEI in the fermented milk, it is necessary to take a considerable amount of fermented milk. Thus, development of a method for producing fermented milk or whey containing a large amount of ACEI has been demanded.

It is known that ACEI such as Val-Pro-Pro and/or Ile-Pro-Pro is highly safe and thus can be used for

pharmaceuticals, functional foods, health foods, and the like. For producing Val-Pro-Pro and/or Ile-Pro-Pro, there is proposed a method including the steps of culturing lactic acid bacteria in a medium containing peptides and/or proteins that have Val-Pro-Pro and/or Ile-Pro-Pro units to prepare fermented milk, and purifying the fermented milk (Japanese Patent No. 2782153).

Conventional lactic acid fermentation, for example for production of typical fermented milk products such as yogurt, is carried out by mixing starter bacteria and a starting material by stirring to form a uniform mixture, and then fermenting the mixture under static conditions in order to make the resulting product as a whole in the form of a curd. Such static conditions are believed to be required because, when a fermentation liquid is at reduced pH due to fermentative proliferation of lactic acid bacteria, application of vibration, such as by stirring or shaking, to such fermentation liquid will cause whey off and coarse texture of the resulting fermented milk products. Further, the lactic acid bacteria for the lactic acid fermentation are facultative anaerobic, so that their growth is often inhibited by oxygen. Accordingly, it has never been intended at all to effect culturing under stirring during the period where the lactic acid fermentation under

static conditions is required. In cheese production, too, the fermentation is carried out by mixing starter bacteria and a starting material by stirring to form a uniform mixture, fermenting the mixture under static conditions, and then coagulating casein by the action of rennet under static conditions, after which the reaction mixture is stirred and pressed for removing whey.

Improvement in whey recovery is required for industrial purification of whey from fermented milk followed by concentration of its active components. A variety of methods for recovering the curd fraction from fermented milk have hitherto been proposed, but effective separation of whey from fermented milk has hardly been performed to date.

Disclosure of the Invention

It is an object of the present invention to provide methods for preparing fermented milk and whey containing an ACEI peptide which enable effective production in high yield of fermented milk and whey having high content of an ACEI peptide that is highly safe and applicable to pharmaceuticals, functional foods, health foods, and the like.

According to the present invention, there is provided a method for producing fermented milk containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced.

According to the present invention, there is also provided a method for producing fermented milk containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated, and

(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced.

According to the present invention, there is further provided a method for producing whey containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed

material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced, and

subjecting the fermented milk to at least one of centrifugation and filter pressing to separate and recover whey.

According to the present invention, there is also provided a method for producing whey containing an ACEI peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated,

(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces and said whey containing the ACEI peptide is produced, and

subjecting the fermented milk to at least one of centrifugation and filter pressing to separate and

recover whey.

Preferred Embodiment of the Invention

The present invention will now be explained in detail.

The present methods include the step of mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material.

The milk as a starting material may be, for example, animal milk such as cow's milk, goat's milk, or sheep's milk; vegetable milk such as soy bean milk; or processed animal or vegetable milk such as skim milk, reconstituted milk, powdered milk, or condensed milk. These may be used as a mixture. Such milk contains peptides and proteins having Val-Pro-Pro and/or Ile-Pro-Pro units.

The solid content of the milk is not particularly limited. For example, when skim milk is used for production of fermented milk, the milk solid-non-fat content thereof is usually about 9 wt%. However, considering the productivity per facility, the milk solid-non-fat content is preferably raised to a certain degree in order to keep the production cost at a lower level. When the lactic acid fermentation under ordinary static conditions only is carried out at the milk solid-non-fat content of 13 wt% or higher, the viscosity of the resulting fermented milk becomes high, which will cause difficulties in separation of whey. Thus, the

milk solid-non-fat content cannot be raised in the ordinary static fermentation. On the contrary, the methods of the present invention keep the resulting fermented milk at low viscosity even at the milk solid-non-fat content of 15 wt% or higher, since the fermentation in the present methods is accompanied by stirring as will be discussed later. Thus, whey can be obtained easily and efficiently.

In the methods of the present invention, the starting material may optionally contain other materials than milk as long as the object of the present invention is achieved. Such other materials may suitably be selected from the materials conventionally used in production of fermented milk, depending on the desired results.

The lactic acid bacteria used in the methods of the present invention are preferably those of the genus Lactobacillus. Examples of such lactic acid bacteria may include *Lactobacillus helveticus*, *Lactobacillus delbruekii* subsp. *bulgaricus*, *Lactobacillus acidophilus*, and the like. In particular, *Lactobacillus helveticus* CM4 (NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN TECHNOLOGY, AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY, Deposit No. FERM BP-6060, Deposition date: August 15, 1997) (referred to as *Lactobacillus helveticus* CM4 hereinbelow) is preferred as ACEI peptide-productive lactic acid bacteria. *Lactobacillus helveticus* CM4

under the deposit number mentioned above has been accepted for deposit under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. All restrictions on the availability to the public of FERM BP-6060 will be irrevocably removed upon the granting of a patent.

In the present invention, the lactic acid bacteria are preferably in the form of a precultured starter having sufficient activity. The initial cell count of the starter is preferably about 10^5 to 10^7 cells/ml.

In the present invention, other microorganisms may optionally be added to the mixed material as long as the object of the present invention is achieved. For example, yeast may additionally be used for improving the flavor and palatability of the resulting fermented milk or whey as functional food, health food, and the like.

Strains of the yeast are not particularly limited, and for example, yeast of the genus Saccharomyces such as *Saccharomyces cerevisiae* may preferably be used. The content of the yeast may suitably be selected, depending on the desired result.

In the methods of the present invention, the mixing by stirring for preparing a mixed material may be conducted by a conventional method so that the lactic

acid bacteria and the starting material are uniformly mixed. Incidentally, this mixing (A) is a conventional step, and distinguished from the fermentation step to be discussed later.

The methods of the present invention include (B-1) fermenting the mixed material under stirring so that curd pieces and whey containing an ACEI peptide are generated, or this fermenting (B-1) and (B-2) fermenting said mixed material under static conditions, whereby fermented milk containing the curd pieces and the whey containing the ACEI peptide is produced.

These steps are for lactic acid fermentation of the mixed material. Conventional lactic acid fermentation has been effected under static conditions so that the mixed material as a whole turns to a lump such as a curd.

In the methods of the present invention, the conditions of the lactic acid fermentation and the final acidity for terminating the fermentation may suitably be set optimally, taking the amount of the ACEI peptide to be generated into account, since the optimum conditions vary depending on the species and strains of lactic acid bacteria, as well as on the milk solid content. For example, when *Lactobacillus helveticus* CM4 is used, the optimum temperature is 25 to 40 ℃, and the duration of the fermentation is about 12 to 40 hours. The final acidity for terminating the fermentation is preferably

about 1.5 to 3 wt % (weight percent of lactic acid).

In step (B-1), the fermentation is effected under stirring. When the lactic acid fermentation is effected only by step (B-1), the fermentation is effected under substantially continuous stirring. On the other hand, when the fermentation is effected by steps (B-1) and (B-2), each of the steps may be conducted at least once, and preferably conducted a plurality of times. In this case, the order of the steps is not particularly limited. The conditions for the stirring, and the conditions for the stirring and the standing may suitably be decided as long as a number of curd pieces and whey containing the ACEI peptide are generated in the fermentation step or steps. Preferably, such conditions may be decided so that the resulting mixture which contains the curd pieces and the whey containing the ACEI peptide has a viscosity of not higher than 20 cp, more preferably not higher than 10 cp. Here, the lower limit of the viscosity is not particularly imposed, but is usually about 2.0 cp. The generation of the curd pieces and whey can be achieved, for example, by setting the conditions so that the stirring is conducted while pH is lowered as the fermentation proceeds from about pH 5, at which soft curds are started to be generated, to pH 4.7-4.6, which is the isoelectric point of casein.

With the conventional fermentation only by

stationary culture, curd is generated in the form of a plain-yogurt-like gel that is substantially contiguous all over the volume of a fermenter (tank). Such fermented milk curd cannot be made into a fermented milk of low viscosity as mentioned above by stirring the curd into pieces after the fermentation. On the contrary, with the methods of the present invention essentially including step (B-1), such a single curd bulk in the form of a contiguous gel is not generated, but curd pieces float, disperse, or precipitate in the whey. The size of the curd pieces may vary depending on various conditions and the kind of the lactic acid bacteria. For example, when the mixed material containing *Lactobacillus helveticus* CM4 is subjected alternately to the fermentation under stirring and the fermentation under static conditions, the size of the curd pieces will be about 3 μm to 5 mm.

In the present invention, the fermentation is preferably effected so that the growth of the lactic acid bacteria is not inhibited by excess oxygen, since the bacteria are facultative anaerobic. Accordingly, the stirring in the fermenting step is preferably carried out so that increase in the amount of oxygen is suppressed that is dissolved in the fermentation liquid due to entrainment of air bubbles therein. For example, the stirring, when continued all through the fermentation,

is preferably carried out at low speed so that the fermentation liquid is softly mixed and fluidized. Specifically, the stirring speed may be about 1 to 50 rpm. Alternatively, when the fermentation is effected by a combination of fermentation under stirring and fermentation under static conditions, i.e., by a combination of steps (B-1) and (B-2), the stirring may be conducted vigorously for a short time to cause entrainment of air bubbles in the fermentation liquid, as long as increase in the amount of oxygen dissolved in the liquid is suppressed.

Surprisingly, by suitably selecting the above stirring conditions, the fermentation under stirring according to the present invention can provide fermented milk containing the ACEI peptide at the same ratio as or even higher ratio than the one produced only by the fermentation under static conditions, as demonstrated in the following Examples.

According to the method of the present invention, fermented milk that contains a large number of curd pieces and whey and that has a low viscosity and excellent workability, can be produced efficiently. Further, whey can also be produced efficiently from such fermented milk through the methods to be discussed later.

In the methods of the present invention, the fermenting steps may be followed by conventional

stirring. In particular, when the fermentation includes step (B-2) of fermenting under static conditions, it is preferred to stir the fermentation product after termination of the fermentation.

The methods for producing whey containing an ACEI peptide of the present invention include, following the above production of the fermented milk, the step of subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey.

The centrifugation of the fermented milk may be carried out in a centrifuge. For example, it is preferred that the centrifugation is carried out continuously at the revolution speed of about 2000 to 10000 rpm. The filter pressing may be carried out in a filter press. It is preferred that the filter pressing is carried out under the pressure of 2 to 8 kg/cm^2.

The fermented milk or whey containing an ACEI peptide obtained by the present methods may be used as fermented milk beverage or milk whey beverage. Further, the whey containing an ACEI peptide may be subjected to treatment such as deacidification, desaltation, concentration, isolation, and the like, for preparation of liquid products; or to drying and powdering treatments for preparation of products in the form of granules or tablets.

Since the methods for producing fermented milk containing an ACEI peptide of the present invention include fermentation under stirring, fermented milk with high ACEI peptide content can be produced efficiently. Further, the methods for producing whey containing an ACEI peptide of the present invention include the steps of fermenting under stirring to prepare fermented milk, and subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey. Thus, whey with high ACEI peptide content can be recovered efficiently. Therefore, these methods facilitate production of products containing an ACEI peptide, and are remarkably effective in industrial point of view.

Examples

The present invention will now be explained in detail with reference to Examples and Comparative Examples. However, the present invention is not limited to these.

Comparative Example 1

900 g of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 9100 g of water, and the resulting solution was subjected to HTST (High Temperature Short Time) pasteurization at 90 ℃ for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a

uniform mixture. The mixture was then fermented under static conditions at 34 ℃ for 25 hours, to thereby obtain fermented milk curd (a) in the form of a contiguous gel with the lactic acid acidity of 2.06 wt %.

Next, the obtained fermented milk curd (a) was stirred and then placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 2.5 kg of whey.

The viscosity and ACEI peptide content of the fermented milk curd (a) were measured under the conditions below. The results are shown in Table 1. Further, the fermented milk curd (a) was stirred, and the particle size of the curd pieces was measured with a particle size analyzer (LA-920 manufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 47 μm, and the arithmetic mean diameter was 27μm.

Viscosity Measurement

The viscosity was measured with VISMETRON viscometer (manufactured by SHIBAURA SYSTEM CO., LTD.) at the liquid temperature of 25 ℃, revolution speed of 60 rpm, using rotor No. 2 for medium viscosity. The duration of measurement was 60 seconds.

Measurement of Val-Pro-Pro and Ile-Pro-Pro Contents

About 1 ml of fermented milk curd (a) as it was, was

placed in an experimental centrifuge, which was operated at 15000 rpm for 10 minutes to collect the supernatant. 0.3 ml of the obtained supernatant was subjected to adsorption on Sep-Pak Cartrige (manufactured by WATERS CO.), followed by washing with distilled water. The adsorbed material was eluted with 5 ml of methanol, and dried in a centrifuging concentrator under reduced pressure. The obtained dried product was dissolved in 0.3 ml of a 0.05 % Trifluoroaceic acid aqueous solution, and analyzed by high performance liquid chromatography (HPLC).

Conditions of Analysis by HPLC

Apparatuses: HITACHI L4000UV DETECTOR
(detection at 215 nm)
L6200 Intelligent pump
L5030 Column Oven (35 ℃)

Conditions of Isolation: Flow Rate at 0.5 ml/min.

Elution Solvent: 0.3 M NaCl, 0.05 % Trifluoroaceic acid aqueous solution

Column: Asahipak GS320 (Φ3.9×600 mm)

ACEI peptide Content: Content of ACEI peptides was calculated by the following formula since Val-Pro-Pro and Ile-Pro-Pro have different ACEI activities:

Content of ACEI peptides (mg/100g)
= Amount of IPP (mg/100g) × 1.7 + Amount of VPP (mg/100g)

Example 1

900 g of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 9100 g of water, and the resulting solution was subjected to HTST pasteurization at 90 ℃ for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 34 ℃ for 29 hours under stirring at 50 rpm, to thereby obtain fermented milk (b) with the lactic acid acidity of 1.88 wt%. The particle size of the curd pieces in the resulting fermented milk (b) was measured with the particle size analyzer (LA-920 manufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 30 μm, and the arithmetic mean diameter was 18 μm.

Next, the obtained fermented milk (b) was placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 6 kg of whey.

The viscosity and ACEI peptide content of the fermented milk (b) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity, for the duration of 30 seconds.

Comparative Example 2

1.5 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 8.5 kg of water, and the resulting solution was subjected to HTST pasteurization at 90℃ for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented under static conditions at 34℃ for 28 hours, to thereby obtain fermented milk curd (c) in the form of a contiguous gel with the lactic acid acidity of 2.81 wt%.

Next, the obtained fermented milk curd (c) was stirred and then placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 100 g of whey.

The viscosity and ACEI peptide content of the fermented milk curd (c) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 3 for high viscosity, for the duration of 60 seconds. The viscosity and ACEI peptide content of the fermented milk curd (c) were measured under the conditions below.

Example 2

1.5 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 8.5 kg of water, and the resulting solution was subjected to HTST pasteurization at 90 ℃ for 1 minute. The pasteurized solution was cooled down to the room temperature, inoculated with 300 g of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 34 ℃ for 30 hours under stirring at 50 rpm, to thereby obtain fermented milk (d) with the lactic acid acidity of 3.04 wt%.

Next, the obtained fermented milk (d) was placed in a centrifuge (manufactured by HITACHI LTD., 20PR52), which was operated at 3000 rpm for 10 minutes to remove curd fraction and recover 6.4 kg of whey.

The viscosity and ACEI peptide content of the fermented milk (d) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity, for the duration of 30 seconds.

Example 3

712 kg of powdered skim milk (manufactured by YOTSUBA MILK PRODUCTS CO., LTD.) was dissolved in 7288 kg of water, and the resulting solution was subjected to plate pasteurization at 92 ℃ and then introduced into a tank

(18000 liter tank manufactured by IWAI KIKAI). The pasteurized solution was cooled down to 35 ℃, inoculated with 240 kg of precultured *Lactobacillus helveticus* CM4, and stirred to make a uniform mixture. The mixture was then fermented at 32 ℃ for 27 hours under intermittent stirring at 50 rpm (by repeating cycles of stirring for 15 minutes and leaving to stand for 45 minutes), to thereby obtain fermented milk (e) with the lactic acid acidity of 1.8 wt%. The particle size of the curd pieces in the resulting fermented milk (e) was measured with the particle size analyzer (LA-920 manufactured by HORIBA LTD.). It was found that 90 % of the curd pieces had a diameter of not larger than 172 μm, and the arithmetic mean diameter was 86 μm.

Next, the obtained fermented milk (e) was placed in a nozzle separator (MBUX510T-34C manufactured by ALFALAVAL, nozzle size 1 mm, flow rate 3500 litter per hour), which was operated at 7490 rpm to recover 6160 kg of whey.

The viscosity and ACEI peptide content of the fermented milk (e) were measured under the same conditions as in Comparative Example 1. The results are shown in Table 1. Incidentally, the viscosity was measured using rotor No. 1 for low viscosity for the duration of 30 seconds.

Table 1

Fermented Milk	Viscosity (cp)	Whey Recovery (%)	ACEI Peptide Content (mg/100g)
Fermented Milk (a) (Comparative Example 1)	415	25	7.1
Fermented Milk (b) (Example 1)	4.5	60	9.0
Fermented Milk (c) (Comparative Example 2)	1832	1	10.5
Fermented Milk (d) (Example 2)	8.1	64	10.8
Fermented Milk (e) (Example 3)	3.8	77	8.6

WHAT IS CLAIMED IS:

1. A method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated,

whereby fermented milk containing said curd pieces and said whey containing the angiotensin converting enzyme inhibitory peptide is produced.

2. A method for producing fermented milk containing an angiotensin converting enzyme inhibitory peptide comprising:

(A) mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material,

(B-1) fermenting said mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated, and

(B-2) fermenting said mixed material under static conditions,

whereby fermented milk containing said curd pieces

and said whey containing the angiotensin converting enzyme inhibitory peptide is produced.

3. The method of claim 1 or 2 wherein said milk is selected from the group consisting of cow's milk, goat's milk, sheep's milk, soy bean milk, skim milk, reconstituted milk, powdered milk, condensed milk and mixtures thereof.

4. The method of claim 1 or 2 wherein said fermented milk has a viscosity of not higher than 20 cp.

5. The method of claim 1 or 2 wherein said angiotensin converting enzyme inhibitory peptide is selected from the group consisting of Val-Pro-Pro, Ile-Pro-Pro, and mixtures thereof.

6. The method of claim 1 or 2 wherein said mixed material further contains a yeast.

7. The method of claim 1 or 2 wherein said lactic acid bacteria contained in the mixed material comprises *Lactobacillus helveticus*.

8. The method of claim 7 wherein said *Lactobacillus helveticus* comprises *Lactobacillus helveticus* CM4

(NATIONAL INSTITUTE OF BIOSCIENCE AND HUMAN TECHNOLOGY, AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY, Deposit No. FERM BP-6060, Deposit date: August 15, 1997).

9. A method for producing whey containing an angiotensin converting enzyme inhibitory peptide comprising:

subjecting the fermented milk produced by the method of claim 1 or 2 to at least one of centrifugation and filter pressing to separate and recover whey, is provided.

ABSTRACT OF THE DISCLOSURE

There are disclosed methods for producing fermented milk and whey that enable effective production in high yield of fermented milk and whey having high content of an ACEI peptide that is highly safe and applicable to pharmaceuticals, functional foods, health foods, and the like. The methods are: a method including the steps of mixing lactic acid bacteria and a starting material containing milk by stirring to prepare a mixed material, and fermenting the mixed material under stirring so that curd pieces and whey containing an angiotensin converting enzyme inhibitory peptide are generated, whereby fermented milk containing the curd pieces and the whey containing the angiotensin converting enzyme inhibitory peptide is produced; and a method including the steps of subjecting the resulting fermented milk to centrifugation and/or filter pressing to separate and recover whey.

Declaration and Power of Attorney For Patent Application

特許出願宣言書

Japanese Language Declaration

私は、下欄に氏名を記載した発明者として、以下のとおり宣言する：

As a below named inventor, I hereby declare that:

私の住所、郵便の宛先および国籍は、下欄に氏名に続いて記載したとおりであり、

My residence, post office address and citizenship are as stated below next to my name,

名称の発明に関し、請求の範囲に記載した特許を求める主題の本来の、最初にして唯一の発明者である（一人の氏名のみが下欄に記載されている場合）か、もしくは本来の、最初にして共同の発明者である（複数の氏名が下欄に記載されている場合）と信じ、

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

PROCESS FOR PRODUCING FERMENTED MILK CONTAINING ANGIOTENSIN CONVERTING ENZYME INHIBITORY PEPTIDE AND PROCESS FOR PRODUCING MILK SERUM

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on January 11, 2000 as an international application under Application Serial No. PCT/JP00/00068

and was amended on _____ (if applicable)

その明細書を

（該当する方に印を付す）

☐ ここに添付する。

☐ _____ 日に出願番号

第 _____ 号として提出し、

_____ 日に補正した。

（該当する場合）

私は、前記のとおり補正した請求の範囲を含む前記明細書の内容を検討し、理解したことを陳述する。

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

私は、連邦規則法典第37部第1章第56条（a）項に従い、本願の審査に所要の情報を開示すべき義務を有することを認める。

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

Japanese Language Declaration

私は、合衆国法典第35部第119 条にもとづく下記の外国特許出願または発明者証出願の外国優先権利益を主張し、さらに優先権の主張に係わる基礎出願の出願日前の出願日を有する外国特許出願または発明者証出願を以下に明記する：

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior foreign applications
先の外国出願
Patent Application

(Number) (番 号)	(Country) (国 名)	(Day/Month/Year Filed) (出願の年月日)	Priority claimed 優先権の主張 Yes あり	No なし
H11-3946	Japan	11 January 1999	☒	☐
			☐	☐
			☐	☐

私は、合衆国法典第35部第120 条にもとづく下記の合衆国特許出願の利益を主張し、本願の請求の範囲各項に記載の主題が合衆国法典第35部第112 条第１項に規定の態様で先の合衆国出願に開示されていない限度において、先の出願の出願日と本願の国内出願日またはＰＣＴ国際出願日の間に公表された連邦規則法典第37部第１章第56条（ａ）項に記載の所要の情報を開示すべき義務を有することを認める：

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (出願番号)	(Filing Date) (出願日)	(現 況) (特許済み、係属中、放棄済み)	(Status) (patented, pending, abandoned)

私は、ここに自己の知識にもとづいて行った陳述がすべて真実であり、自己の有する情報および信ずるところに従って行った陳述が真実であると信じ、さらに故意に虚偽の陳述等を行った場合、合衆国法典第18部第1001条により、罰金もしくは禁錮に処せられるか、またはこれらの刑が併科され、またかかる故意による虚偽の陳述が本願ないし本願に対して付与される特許の有効性を損うことがあることを認識して、以上の陳述を行ったことを宣言する。

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Japanese Language Declaration

委任状：私は、下記発明者として、以下の代理人をここに選任し、本願の手続を遂行すること並びにこれに関する一切の行為を特許商標庁に対して行うことを委任する。
（代理人氏名および登録番号を明記のこと）

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (*list name and registration number*)

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（第六またはそれ以降の共同発明者に対しても同様な情報および署名を提供すること。）

(Supply similar information and signature for third and subsequent joint inventors.)